DOI 10.1007/s10593-021-02926-2
Chemistry of Heterocyclic Compounds **2021**, *57*(4), 473–478

Methods of synthesis and antiviral activity of new 4-alkyl-3-nitro-1,4-dihydroazolo[5,1-*c*][1,2,4]triazin-4-ols

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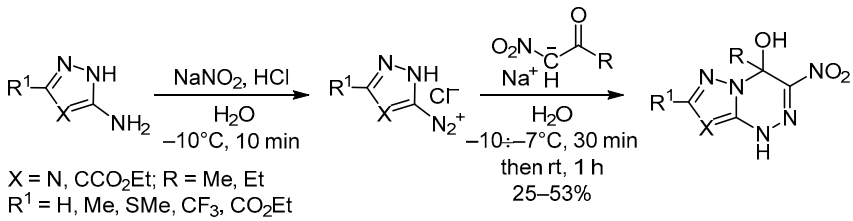
Translated from Khimiya Geterotsiklicheskikh Soedinenii, 2021, *57*(4), 473–478

Submitted November 11, 2020
Accepted after revision December 30, 2020



An azo coupling reaction of α-nitro ketones with 5-diazoazoles was used to obtain 4-alkyl-3-nitro-1,4-dihydroazolo[5,1-*c*][1,2,4]triazines, which were characterized with respect to their antiviral activity against influenza and Coxsackie B3 viruses.

Keywords: 3-nitroazolo[5,1-*c*][1,2,4]triazines, α-nitro ketones, triazines, antiviral activity, Coxsackie B3 virus, influenza virus.

Recent challenges to the humanity include new viral diseases that are caused, for example, by a previously unknown human metapneumovirus, novel types of coronaviruses, and the influenza viruses.[1,2] Influenza and acute respiratory viral infections account for approximately 90% of infectious respiratory pathologies in humans. The annual case load due to these diseases in the Russian Federation ranges from 18 to 33 million patients. Among them, influenza represents the greatest concern because of its rapid transmission, severe cource of disease, and the probability of occurrence of serious complications. Specific treatments of influenza rely on drugs belonging to several chemical categories, differing by their molecular targets and the mechanism of action. Prominent examples include the M2 ion channel inhibitors rimantadine and amantadine, the neuraminidase inhibitors oseltamivir, zanamivir, peramivir, and laninamivir,[3] as well as the viral polymerase complex blocker baloxavir marboxil.[4] Umifenovir (Arbidol) is a viral hemagglutinin blocker that has been approved for use in Russia, China, and several other countries.[5] However, the properties of influenza virus genome enable rapid emergence of strains resistant to the action of known antiviral drugs, while the virus retains its virulence and pathogenicity.[6] A series of nucleoside analogs are effective against influenza. For example, favipiravir is a broad spectrum antiviral drug developed in Japan.[7] The well-known drug ribavirin, which also belongs to this group, shows anti-influenza properties as well. However, these drugs are not frequently prescribed against influenza due to their significant side effects.

Pathogens belonging to the genus of enteroviruses, such as Coxsackie B viruses, are also the source of major human health concerns. These non-enveloped viruses containing positive-sense RNA genome are the causative agents for a broad spectrum of pathologies, including pericarditis and myocarditis, poliomyelitis, nonspecific febrile illness, serous meningitis, meningoencephalitis, and other diseases. Myocarditis associated with Coxsackie virus infection is

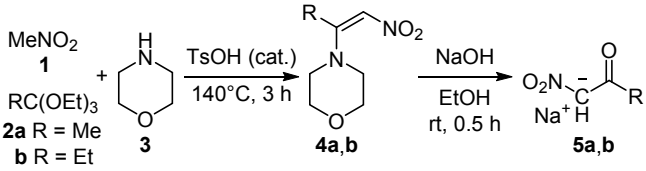
exceedingly difficult for diagnosis and treatment, due to both clinical issues (a variable degree of organic and functional damage to the myocardium, difficulty in obtaining biopsy samples, and the absence of clear disease markers), as well as taking into account the absence of effective agents for etiotropic treatment.[8] Taking into account all of the aforementioned factors, it should be recognized that the development of new, effective antiviral agents, including drugs against Coxsackie B3 virus, is a high priority task for medicinal chemistry and practical healthcare.

Azolo[5,1-*c*][1,2,4]triazines are heterocyclic systems that possess a range of useful biological properties.[9] The steady interest of researchers toward such compounds is primarily motivated by their structural similarity to the heterocyclic nucleobases of DNA and RNA. Such structural characteristics are associated with antimetabolite activity, which can be instrumental for designing effective biologically active compounds. A particular interest in this area has been attracted by compounds bearing a nitro group in the [1,2,4]triazine ring, which have shown a broad range of antiviral activity. This class of compounds is represented by Triazavirin (6-methylsulfanyl-3-nitro[1,2,4]triazolo[5,1-*c*]-[1,2,4]triazin-4-one sodium salt dihydrate), which has been approved for human use in the treatment of influenza, acute respiratory viral infections, and tick-borne encephalitis.[10–15]

The syntheses of azolo[1,2,4]triazine ring systems have been accomplished according to two main approaches: the annulation of an azole ring to an existing [1,2,4]triazine ring or the assembly of a triazine moiety on the basis of azole-containing starting material.[16] Nitro synthons have been of particular interest in the synthesis of azolo[1,2,4]triazines. Suitable nitro components that have been used in these syntheses are ethyl nitroacetate,[9] ethyl nitromalonate,[17] nitroacetonitrile,[18–20] nitromalonic aldehyde,[17] and nitroacetaldehyde in the form of its potassium salt.[21]

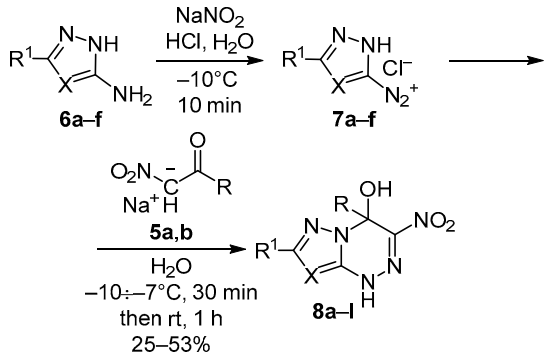
Promising synthetic building blocks for the assembly of triazine ring include α-nitro ketones, which are highly reactive compounds with a broad synthetic potential but low thermodynamic stability, which is a drawback for their synthetic applications. In our earlier study we have described a new, effective method for the synthesis of α-nitro ketone salts **5a**,**b** from nitroenamines **4a**,**b** that, in turn, were obtained from nitromethane (**1**), ortho esters **2a**,**b**, and morpholine (**3**) (Scheme 1).[22,23]

Scheme 1



Azo coupling of 1-nitro-2-propanone sodium salt (**5a**) or 1-nitro-2-butanone sodium salt (**5b**) with 5-diazoazoles **7a–f** obtained *in situ* from amines **6a–f** led to the formation of new 4-methyl-3-nitro-1,4-dihydroazolo[5,1-*c*][1,2,4]triazin-4-ols **8a–f** and 4-ethyl-3-nitro-1,4-dihydroazolo[5,1-*c*]-[1,2,4]triazin-4-ols **8g–l** in 25–53% yields (Scheme 2).

Scheme 2



8 a X = N, R^1 = H, R = Me; **b** X = N, R^1 = Me, R = Me;
c X = N, R^1 = SMe, R = Me; **d** X = N, R^1 = CF_3, R = Me;
e X = N, R^1 = CO_2Et, R = Me; **f** X = CCO_2Et, R^1 = H, R = Me;
g X = N, R^1 = H, R = Et; **h** X = N, R^1 = Me, R = Et;
i X = N, R^1 = SMe, R = Et; **j** X = N, R^1 = CF_3, R = Et;
k X = N, R^1 = CO_2Et, R = Et; **l** X = CCO_2Et, R^1 = H, R = Et

Only isolated examples of alkyl-1,4-dihydroazolo[1,2,4]-triazin-4-ols have been described in the literature. Similar compounds have been obtained using 3-fluoroalkyl-3-oxopropanoate and its analogs,[24,25] which are costly compounds with limited availability. The described 4-fluoroalkyl-1,4-dihydroazolo[5,1-*c*][1,2,4]triazin-4-ols were characterized with respect to their inhibitory activity against mixed type carboxylesterase.[24]

The structures of 4-alkylazolo[5,1-*c*][1,2,4]triazin-4-ols **8a–l** were characterized by IR, ^{1}H and ^{13}C NMR spectroscopy, as well as by elemental analysis. A crystal of compound **8j** (Fig. 1) was subjected to X-ray structural analysis (Fig. 1). ^{1}H NMR spectra of 4-alkylazolo[5,1-*c*]-[1,2,4]triazin-4-ols **8a–l** contained characteristic upfield signals of alkyl group at position 4 of the triazine ring, as well as a hydroxy group singlet in the region of 5.97–7.92 ppm. ^{13}C NMR spectra of compounds **8a–l** also contained characteristic upfield signals of alkyl group at the triazine ring position 4, as well as signals assigned to the azole ring substituents. Characteristic IR absorption bands in the regions of 1530–1580 and 1316–1338 cm^{-1} belonged to the nitro group.

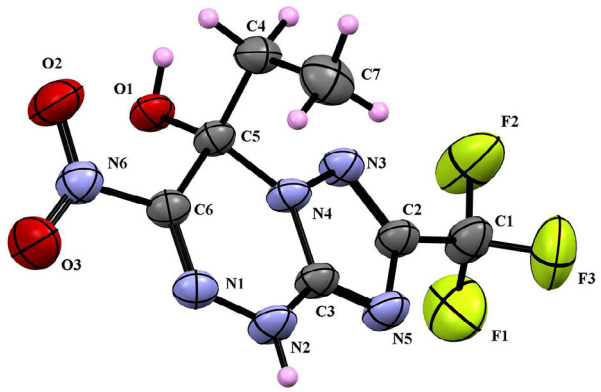
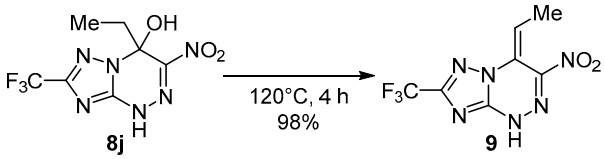


Figure 1. The molecular structure of compound **8j** with atoms represented by thermal vibration ellipsoids of 50% probability.

In the case of compound **8j**, it was shown that compounds belonging to the 4-alkyl-3-nitro-1,4-dihydroazolo-[5,1-*c*][1,2,4]triazin-4-ol series can undergo the elimination of H_2O molecule upon gradual heating, forming the conjugated triazolotriazine system **9** (Scheme 3). The structure of compound **9** was confirmed by ^{1}H NMR spectroscopy data. Its characteristic ^{1}H NMR features included a doublet at 2.27 ppm, which was assigned to the methyl group, and a quartet signal of the methylene moiety at 5.76 ppm. Compound **9** was also characterized by IR spectroscopy, ^{13}C NMR spectroscopy, and elemental analysis.

Scheme 3



Some of the synthesized series of compounds **8a**–**l** were characterized with respect to their biological activity. Thus, their cytotoxic properties were determined in a cell culture, as well as their inhibitory activity was determined against two phylogenetically unrelated viruses – the influenza virus and Coxsackie B3 virus (Table 1).

According to the obtained data, the studied compounds exhibited a broad range of toxicity. The most toxic was compound **8f** (CC_{50} 139 μM), while the least toxic was compound **8b**, which did not cause cell death up to the highest concentrations tested (300 μg/ml, 1415 μM). Despite the small number of compounds that were tested, a trend of increased toxicity in compounds of this group can be associated with the presence of methylsulfanyl or trifluoro-methyl substituent in the azole ring.

Table 1. The cytotoxic and antiviral properties of 4-alkyl-3-nitro-1,4-dihydroazolo[5,1-*c*][1,2,4]triazines **8a**–**l** in cell cultures

Compound	Influenza virus (MDCK cells)			Coxsackie B3 virus (Vero cells)		
	CC_{50}*, μM	IC_{50}**, μM	SI***	CC_{50}*, μM	IC_{50}**, μM	SI***
8a	N/T*[4]	N/T	–	N/T	N/T	–
8b	>1415	472±30	3	N/T	N/T	–
8c	N/T	N/T	–	N/T	N/T	–
8d	N/T	N/T	–	N/T	N/T	–
8e	371 ± 22	>370	1	N/T	N/T	–
8f	139 ± 11	91 ± 12	2	N/T	N/T	–
8g	742 ± 51	>469	2	390	>235	2
8h	907 ± 78	233 ± 29	4	436	>220	2
8i	419 ± 36	32 ± 4	13	368	>194	2
8j	432 ± 28	>357	1	425	129	3
8k	>1056	>1056	1	N/T	N/T	–
8l	1060 ± 55	336 ± 40	3	777	>353	2
Ribavirin	>2130	39 ± 5	55	>2130	56 ± 8	38

* CC_{50} – 50% cytotoxic concentration – the concentration of test compound resulting in death of 50% of cells.
** IC_{50} – 50% inhibitory concentration – the concentration of test compound reducing the virus titer by 50%, compared to the blank control.
*** SI – selectivity index – the ratio of CC_{50} to IC_{50}.
*[4] N/T (Not Tested) – not determined.

Among all of the obtained representatives, only compound **8i** containing a methylsulfanyl substituent showed a moderate antiviral activity against the influenza virus. The other compounds suppressed the viral reproduction to a certain degree only at nearly toxic concentrations. It should be noted that the known antiviral drug Triazavirin, belonging to the class of azoloazines, also contains a methylsulfanyl moiety. Apparently, this structural motif should be viewed as an important promoter of anti-influenza activity in the case of azoloazines, providing guidance for further optimization and focusing of chemical libraries. Regarding the phylogenetically distant Coxsackie B3 virus, none of the analyzed candidates, including compound **8i**, showed inhibiting activity. The IC_{50} values in this case were on the range of hundreds of μM and were close to the toxicity limit for these compounds. This observation should be interpreted as evidence of a specific mechanism of action for compound **8i** against the influenza virus, explained by the presence of a particular functionality.

Thus, we have synthesized a series of 4-alkyl-3-nitro-1,4-dihydroazolo[5,1-*c*][1,2,4]triazines by using α-nitro ketone salts. The obtained compounds were characterized with respect to their cytotoxic properties in cell culture, as well as their inhibitory activity against phylogenetically unrelated viruses – the influenza virus and Coxsackie B3 virus. The obtained results and literature data indicate that azolo[5,1-*c*][1,2,4]triazines are promising objects for further medicinal chemistry research.

Experimental

IR spectra were recorded on a Bruker Alpha spectrometer equipped with a ZnSe ATR accessory. ^{1}H, ^{13}C, and ^{19}F NMR spectra were recorded on a Bruker Avance NEO 600 instrument (600, 151, and 565 MHz, respectively) for samples in CD_3CN and Me_2CO-d_6 solutions, with TMS used as internal standard. Elemental analyses were performed on a PerkinElmer 2400 Series II CHNS elemental analyzer. Melting points were determined on an Electrothermal IA 9100 melting point apparatus. The reaction progress and purity of the obtained compounds were controlled by TLC using Silufol UV-254 plates.

This study was performed using commercially available reagents and solvents without additional purification, unless indicated otherwise.

Preparation of 3-nitro-1,4-dihydroazolo[5,1-*c*][1,2,4]-triazin-4-ols 8a–l (General method). A solution of $NaNO_2$ (0.76 g, 0.011 mol) in H_2O (2 ml) was added portionwise to a solution of 3-aminoazole **6a**–**f** (0.01 mol) in H_2O (4 ml) and concd HCl (6 ml, 0.072 mol) at –10÷–7°C. The reaction mixture was stirred for 10 min and treated by adding a solution of 1-nitropropan-2-one sodium salt monohydrate (**5a**) (0.01 mol) or 1-nitrobutan-2-one sodium salt (**5b**) (0.01 mol) in H_2O (6 ml). The mixture was stirred

for 30 min at –10÷–7°C and then for 1 h at room temperature. The product was extracted with EtOAc, the solvent was evaporated, the residue was stirred with $CHCl_3$ (20 ml) for 30 min and filtered off, then washed with EtOH.

4-Methyl-3-nitro-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]-triazin-4-ol (8a). Yield 0.71 g (36%), yellow powder, mp 146–148°C (EtOAc). IR spectrum, ν, cm^{-1}: 3223 (O–H), 3141 (N–H), 1530, 1320 (NO_2), 1206, 1184 (C–O). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm: 2.40 (3H, s, CH_3); 7.03 (1H, s, CH); 7.86 (1H, s, OH); 12.12 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 25.5; 83.2; 144.4; 146.5; 152.1. Found, %: C 30.69; H 3.05; N 42.62. $C_5H_6N_6O_3$. Calculated, %: C 30.31; H 3.05; N 42.21.

4,7-Dimethyl-3-nitro-1,4-dihydro[1,2,4]triazolo[5,1-*c*]-[1,2,4]triazin-4-ol (8b). Yield 0.53 g (25%), yellow powder, mp 151–153°C (decomp.). IR spectrum, ν, cm^{-1}: 3264 (O–H), 3100 (N–H), 1556, 1318 (NO_2), 1174 (C–O). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm: 2.26 (3H, s, CH_3); 2.36 (3H, s, CH_3); 6.89 (1H, s, OH); 11.98 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 14.5; 25.6; 82.9; 144.5; 146.7; 161.4. Found, %: C 33.91; H 3.81; N 39.54. $C_6H_8N_6O_3$. Calculated, %: 33.97; H 3.80; N 39.61.

4-Methyl-7-(methylsulfanyl)-3-nitro-1,4-dihydro[1,2,4]-triazolo[5,1-*c*][1,2,4]triazin-4-ol (8c). Yield 0.63 g (26%), yellow powder, mp 151–153°C (EtOAc). IR spectrum, ν, cm^{-1}: 3250 (O–H), 3168 (N–H w), 1556, 1316 (NO_2), 1157 (C–O). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm: 2.37 (3H, s, CH_3); 2.56 (3H, s, SCH_3); 6.99 (1H, s, OH); 12.07 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 14.0; 25.4; 83.1; 144.7; 147.2; 162.8. Found, %: C 29.51; H 3.18; N 34.05. $C_6H_8N_6O_3S$. Calculated, %: C 29.51; H 3.30; N 34.41.

4-Methyl-3-nitro-7-(trifluoromethyl)-1,4-dihydro[1,2,4]-triazolo[5,1-*c*][1,2,4]triazin-4-ol (8d). Yield 0.85 g (32%), yellow powder, mp 164–166°C (EtOAc). IR spectrum, ν, cm^{-1}: 3329 (O–H), 3196 (N–H), 1539, 1329 (NO_2), 1152 (C–F). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm: 2.43 (3H, s, CH_3); 7.37 (1H, s, OH); 12.41 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 25.4; 84.2; 120.2 (q, *J* = 269.2); 144.8; 147.8; 153.5 (q, *J* = 39.6). ^{19}F NMR spectrum (Me_2CO-d_6), δ, ppm: –66.65 (CF_3). Found, %: C 27.14; H 1.66; N 31.55. $C_6H_5N_6O_3F_3$. Calculated, %: C 27.08; H 1.89; N 31.58.

Ethyl 4-hydroxy-4-methyl-3-nitro-1,4-dihydro[1,2,4]-triazolo[5,1-*c*][1,2,4]triazine-7-carboxylate (8e). Yield 1.11 g (41%), yellow powder, mp 194–196°C (EtOAc). IR spectrum, ν, cm^{-1}: 3272 (O–H), 1676 (C=O), 1579, 1322 (NO_2), 1148 (C–O). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 1.37 (3H, t, *J* = 7.1, CH_3); 2.44 (3H, s, CH_3); 4.35–4.44 (2H, m, CH_2); 7.22 (1H, s, OH); 12.28 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 14.5; 25.6; 62.3; 84.1; 144.6; 147.2; 154.8; 160.1. Found, %: C 35.40; H 3.90; N 31.32. $C_8H_{10}N_6O_5$. Calculated, %: C 35.56; H 3.73; N 31.10.

Ethyl 4-hydroxy-4-methyl-3-nitro-1,4-dihydropyrazolo-[5,1-*c*][1,2,4]triazine-8-carboxylate (8f). Yield 1.34 g (50%), yellow powder, mp 153–155°C (EtOAc). IR spectrum, ν, cm^{-1}: 3272 (O–H), 1676 (C=O), 1579, 1322 (NO_2), 1148 (C–O). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 1.32 (3H, t, *J* = 7.1, CH_3); 2.42 (3H, s, CH_3); 4.32 (2H, q, *J* = 7.1, CH_2); 6.96 (1H, s, CH); 7.90 (1H, s, OH); 11.48 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 14.9; 25.2; 60.9; 81.8; 97.4; 138.6; 142.1; 144.9; 162.7. Found, %: C 39.64; H 3.92; N 25.81. $C_9H_{11}N_5O_5$. Calculated, %: C 40.15; H 4.12; N 26.01.

4-Ethyl-3-nitro-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]-triazin-4-ol (8g). Yield 1.09 g (51%), yellow powder, mp 147–149°C (EtOAc). IR spectrum, ν, cm^{-1}: 1540, 1321 (NO_2), 1029 (C–O). ^1H NMR spectrum (CD_3CN), δ, ppm (*J*, Hz): 0.65 (3H, t, *J* = 7.5, CH_3); 2.68–2.80 (2H, m, CH_2); 5.72 (1H, s, CH); 7.84 (1H, s, OH); 10.82 (1H, s, NH). ^{13}C NMR spectrum (CD_3CN), δ, ppm: 8.7; 31.1; 87.9; 143.4; 147.4; 152.5. Found, %: C 33.80; H 3.73; N 39.26. $C_6H_8N_6O_3$. Calculated, %: C 33.97; H 3.80; N 39.61.

4-Ethyl-7-methyl-3-nitro-1,4-dihydro[1,2,4]triazolo-[5,1-*c*][1,2,4]triazin-4-ol (8h). Yield 0.98 g (43%), yellow powder, mp 163–165°C (EtOAc). IR spectrum, ν, cm^{-1}: 1545, 1330 (NO_2 s), 1132 (C–O s). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 0.71 (3H, t, *J* = 7.5, CH_3); 2.27 (3H, s, CH_3); 2.74–2.88 (2H, m, CH_2); 6.98 (1H, s, OH); 12.04 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 8.6; 14.4; 30.9; 87.3; 142.9; 147.2; 162.1. Found, %: C 37.06; H 4.54; N 37.22. $C_7H_{10}N_6O_3$. Calculated, %: C 37.17; H 4.46; N 37.15.

4-Ethyl-7-(methylsulfanyl)-3-nitro-1,4-dihydro[1,2,4]-triazolo[5,1-*c*][1,2,4]triazin-4-ol (8i). Yield 1.18 g (46%), yellow powder, mp 151–153°C (EtOAc). IR spectrum, ν, cm^{-1}: 1539, 1326 (NO_2 s), 1133 (C–O s). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 0.75 (3H, t, *J* = 7.5, CH_3); 2.56 (3H, s, SCH_3); 2.73–2.89 (2H, m, CH_2); 7.05 (1H, s, OH); 12.10 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 8.8; 14.1; 30.9; 87.9; 143.7; 148.1; 163.3. Found, %: C 32.60; H 3.93; N 32.56. $C_7H_{10}N_6O_3S$. Calculated, %: C 32.55; H 3.90; N 32.54.

4-Ethyl-3-nitro-7-(trifluoromethyl)-1,4-dihydro[1,2,4]-triazolo[5,1-*c*][1,2,4]triazin-4-ol (8j). Yield 1.50 g (53%), yellow powder, mp 171–173°C (EtOAc). IR spectrum, ν, cm^{-1}: 3431 (O–H), 1553, 1338 (NO_2), 1157 (C–F), 1133 (C–O). ^1H NMR spectrum (CD_3CN), δ, ppm (*J*, Hz): 0.70 (3H, t, *J* = 7.5, CH_3); 2.68–2.78 (2H, m, CH_2); 5.97 (1H, s, OH); 10.97 (1H, s, NH). ^{13}C NMR spectrum (CD_3CN), δ, ppm (*J*, Hz): 8.6; 31.3; 88.8; 120.1 (q, *J* = 270.3); 143.8; 148.7; 153.9 (q, *J* = 39.3). ^{19}F NMR spectrum (CD_3CN), δ, ppm: –67.13 (CF_3). Found, %: C 29.91; H 2.48; N 30.00. $C_7H_7N_6O_3F_3$. Calculated, %: C 30.01; H 2.52; N 30.00.

Ethyl 4-ethyl-4-hydroxy-3-nitro-1,4-dihydro[1,2,4]-triazolo[5,1-*c*][1,2,4]triazine-7-carboxylate (8k). Yield 1.13 g (40%), yellow powder, mp 189–191°C (EtOAc). IR spectrum, ν, cm^{-1}: 1748 (C=O), 1540, 1328 (NO_2), 1033 (C–O). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 0.75 (3H, t, *J* =7.5, CH_3); 1.37 (3H, t, *J* = 7.1, CH_3); 2.82–2.92 (3H, m, CH_3); 4.35–4.44 (2H, m, CH_2); 7.37 (1H, s, OH); 12.35 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 8.7; 14.5; 31.4; 62.4; 88.7; 143.5; 148.1; 155.2; 160.0. Found, %: C 38.01; H 4.11; N 29.61. $C_9H_{12}N_6O_5$. Calculated, %: C 38.03; H 4.26; N 29.57.

Ethyl 4-ethyl-4-hydroxy-3-nitro-1,4-dihydropyrazolo-[5,1-*c*][1,2,4]triazine-8-carboxylate (8l). Yield 1.34 g (50%),

yellow powder, mp 184–186°C (EtOAc). IR spectrum, ν, cm^{-1}: 3256 (O–H), 3128 (N–H), 1682 (C=O), 1580, 1317 (NO_2), 1156, 1116 (C–O). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 0.69 (3H, t, *J* = 7.5, CH_3); 1.33 (3H, t, *J* = 7.1, CH_3); 2.78–3.02 (2H, m, CH_2); 4.29–4.37 (2H, m, CH_2); 7.05 (1H, s, CH); 7.92 (1H, s, OH); 11.52 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm: 8.8; 14.8; 30.7; 60.9; 86.5; 97.4; 139.4; 142.5; 143.7; 162.6. Found, %: C 42.06; H 4.57; N 23.68. $C_{10}H_{13}N_5O_5$. Calculated, %: C 42.40; H 4.63; N 24.73.

4-Ethylidene-3-nitro-7-(trifluoromethyl)-1,4-dihydro-[1,2,4]triazolo[5,1-*c*][1,2,4]triazine (9). 4-Ethyl-3-nitro-7-(trifluoromethyl)-1,4-dihydro[1,2,4]triazolo[5,1-*c*][1,2,4]-triazin-4-ol **(8j)** 0.28 g (0.001 mol) was heated at 120°C without solvent and maintained for 4 h. Yield 0.26 g (98%), brown powder, mp 174–176°C. IR spectrum, ν, cm^{-1}: 1540, 1362 (NO_2), 1148 (C–F). ^1H NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 2.27 (3H, d, *J* = 7.7, CH_3); 5.76 (1H, q, *J* = 7.7, CH); 12.17 (1H, s, NH). ^{13}C NMR spectrum (Me_2CO-d_6), δ, ppm (*J*, Hz): 12.9; 111.2; 119.9 (q, *J* = 269.3); 121.1; 147.6; 149.6; 153.4 (q, *J* = 39.4). ^{19}F NMR spectrum (C_3D_6O), δ, ppm: –66.88 (3F, CF_3). Found, %: C 32.04; H 1.74; N 31.92. $C_7H_5N_6O_2F_3$. Calculated, %: C 32.07; H 1.92; N 32.06.

Cytotoxic activity study of compounds 8a–l in cell culture was performed on the basis of cell viability evaluation using the reduction reaction of tetrazolium dye MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) by cells in the culture, the intensity of which reflected the degree of cell viability as indicated by the reduction of dye by cellular dehydrogenases.

The influenza virus strain A/Puerto Rico/8/34 (H1N1) was cultured in MDCK cells, while Coxsackie B3 virus was cultured in Vero cells. The compounds under investigation dissolved at the concentration range of 4–300 μg/ml (for the influenza virus) and 12.5–400 μg/ml (for the Coxsackie B3 virus) in the cell culture medium, were added into the wells of a 96-well plate with a monolayer of cells at the volume of 200 μl/well, followed by incubation for 48 h at 37°C at 5% CO_2. Cells were further washed with α-MEM medium and 100 μl of MTT solution (0.5 mg/ml) in cell culture medium was added to each well of the plate. The cells were incubated at 37°C at 5% CO_2 for 2 h and washed for 5 min with saline. The precipitate was dissolved in DMSO (100 μl in each well of the plate), followed by optical density measurement using a Thermo Scientific Multiskan FC microplate reader at 540 nm wavelength. The 50% cytotoxic concentration (CC_{50}) was calculated on the basis of the obtained data, reflecting the concentration of the compound that reduced the optical density in the wells by 50%, compared to intact cells.

Antiviral activity testing of compounds 8a–l. The antiviral activity was determined against the A/Puerto Rico/8/34 (H1N1) strain of influenza virus and the Coxsackie B3 virus (strain Nancy). The solutions of compounds under investigation dissolved at the concentration range of 4–300 μg/ml (for the influenza virus) and 12.5–400 μg/ml (for the Coxsackie B3 virus) in the cell culture medium (100 μl) were added into the plate wells containing a monolayer of MDCK cells for influenza virus or Vero cells for Coxsackie B3 virus. The plates with cells were incubated at 5% CO_2 at 37°C for 1 h. Subsequently, the respective virus (100 μl) was added to the wells (at m.o.i. 0.01), and the plates were incubated for 48 h at 5% CO_2 at 37°C. After the incubation period, the cells were washed with α-MEM medium and cell viability was determined, as described before. On the basis of the obtained data, the 50% inhibitory concentration (IC_{50}) was calculated as the concentration of a compound providing 50% reduction in the cell death caused by the virus.

Data analysis. The values of 50% cytotoxic concentration (CC_{50}) and 50% effective concentration (IC_{50}) were calculated using the GraphPad Prism 6.01 software suite. The working model selected for the analysis was a 4-parameter logistic curve equation (choosing nonlinear analysis for finding the logarithm of inhibitor). On the basis of the obtained data, the selectivity index (SI) was calculated for each compound against each virus as the ratio of CC_{50} to IC_{50}. Those compounds for which SI is 10 and higher are considered promising.

X-ray structural analysis of compound 8j was performed on an automatic Xcalibur 3 diffractometer according to the standard methodology: MoKα radiation, λ 0.71073 Å, graphite monochromator, ω-scanning with a step of 1° at 292(2)K. Empirical correction for absorption was introduced. The structure was solved by a direct statistical method and refined by full-matrix method of least squares by F^2 in ansiotropic approximation for all non-hydrogen atoms. The positions of hydrogen atoms linked by C–H bonds were calculated geometrically, the positions of OH and NH protons were refined independently in isotropic approximation. All calculations were performed by using the SHELXTL software suite.[26] The main crystallographic parameters for the compound: triclinic syngony, space group $P\bar{1}$; *a* 6.2042(6), *b* 8.5391(7), *c* 10.6202(9) Å; α 85.326(7), β 77.235(8), γ 76.542(8)°; μ 0.170 mm^{-1}. A total of 4608 reflections were collected for angles $3.60 < \theta < 30.83°$, of which 2874 were independent reflections (R_{int} 0.0318). The final refinement parameters: R_1 0.0972, wR_2 0.2003 (by all reflections), R_1 0.0585, wR_2 0.1536 (by reflections with $I > 2\sigma(I)$) at the reliability factor GOOF 1.004. The peaks of residual electron density were 0.280/–0.365 $eÅ^{-3}$. The complete X-ray diffraction analysis dataset for compound **8j** was deposited at the Cambridge Crystallographic Data Center (deposit CCDC 2042591).

Supplementary information file containing ^1H, ^{13}C, and ^{19}F NMR spectra of the synthesized compounds is available at the journal website at http://link.springer.com/journal/10593.

This study was funded by the Russian Science Foundation (project No. 20-13-00142).

X-ray structural analysis was performed on the equipment at the Collective Use Center of I. Ya. Postovsky Institute of Organic Synthesis, the Ural Branch of the Russian Academy of Sciences. Spectral data were obtained

on the equipment of the laboratory "Comprehensive research and expert assessment of organic materials" .

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